



Tracking Particle-Encapsulated DNA Across the Anion-Exchange Chromatography Fractions of Recombinant Adeno-Associated Virus Using Droplet Digital PCR and High-Throughput...

Hashiba, Noriko ; Yuan, Yuzhe ; Itou-Kudo, Emi ; Masumi-Koizumi, Kyoko ; Yusa, Keisuke ; Uchida, Kazuhisa

(Citation)

Biotechnology Journal, 20(6):e70031

(Issue Date)

2025-06

(Resource Type)

journal article

(Version)

Version of Record

(Rights)

© 2025 The Author(s). Biotechnology Journal published by Wiley-VCH GmbH.
This is an open access article under the terms of the Creative Commons Attribution-NonCommercial License, which permits use, distribution and reproduction in any medium, provided the original work is properly cited and is not used for commercial purposes.

(URL)

<https://hdl.handle.net/20.500.14094/0100496279>



RESEARCH ARTICLE OPEN ACCESS

Tracking Particle-Encapsulated DNA Across the Anion-Exchange Chromatography Fractions of Recombinant Adeno-Associated Virus Using Droplet Digital PCR and High-Throughput Sequencing

Noriko Hashiba^{1,2} | Yuzhe Yuan¹ | Emi Itou-Kudo¹ | Kyoko Masumi-Koizumi¹ | Keisuke Yusa¹ | Kazuhisa Uchida^{1,2} 

¹Graduate School of Science, Technology and Innovation, Kobe University, Kobe, Japan | ²Biologics Center for Research and Training, Tokyo, Japan

Correspondence: Kazuhisa Uchida (kazuhisa.uchida@port.kobe-u.ac.jp)

Received: 21 October 2024 | **Revised:** 11 April 2025 | **Accepted:** 22 April 2025

Funding: K.U. is supported by grant from the Japan Agency for Medical Research and Development (AMED; JP18ae0201001).

Keywords: AEX fractions | ddPCR | high-throughput sequencing | rAAV genomic and impurity DNA | recombinant adeno-associated virus

ABSTRACT

Introduction: Safe and effective adeno-associated virus (AAV) vectors are essential for gene therapy. Particle heterogeneity, specifically particle DNA of varying types and sizes, significantly affects recombinant adeno-associated virus (rAAV) performance. Previous studies have identified particle-associated DNAs; however, the specific DNA composition of these mixed populations remains poorly understood. This study aimed to investigate the DNA composition of the isolated subpopulations of rAAV particles obtained through anion exchange (AEX) chromatography.

Methods: RAAV2-ZsGreen1 particles were fractionated on an AEX column, resulting in 12 distinct fractions. We analyzed the DNA composition of these fractions using droplet digital PCR (ddPCR) and MiSeq to identify the incorporated DNA heterogeneity in them.

Results: Our findings illustrated a clear trend in which the DNA content increasing in fractions was associated with an increased rAAV genomic DNA ratio of total particle DNA. The particle DNA content increased significantly across fractions from Peak 1 to Peak 2, showing approximately 30,000- and 5000-fold increases for ZsGreen1 (rAAV genome) and *amp^R* (plasmid impurity), respectively. Notably, in the empty particle subpopulations, the rate of detectable DNA molecules was lower than one DNA fragment per 100 particles, with inverted terminal repeat (ITR) sequences being the most prevalent.

Conclusions: With the elucidated profile of particle DNAs, this study provides detailed information on particle heterogeneity, shedding light on empty and partial particle formation and impurity DNA incorporation.

1 | Introduction

Recombinant adeno-associated virus (rAAV) is a common gene therapy vector known for its ability to readily guide viral tropism to the target tissues and organs [1]. However, the purity of

the packaged DNA within rAAV particles directly impacts gene transfer efficiency and potential side effects [2, 3]. This DNA inclusion is initiated with Rep binding to Rep-binding element (RBE) sequences in the inverted terminal repeat (ITR) regions, plasmids, or host cell genomes. Consequently, rAAV production

Abbreviations: AAV, adeno-associated virus; AEX, anion exchange; ddPCR, droplet digital PCR; HTS, high-throughput sequencing; rAAV, recombinant adeno-associated virus.

This is an open access article under the terms of the [Creative Commons Attribution-NonCommercial](https://creativecommons.org/licenses/by-nc/4.0/) License, which permits use, distribution and reproduction in any medium, provided the original work is properly cited and is not used for commercial purposes.

© 2025 The Author(s). *Biotechnology Journal* published by Wiley-VCH GmbH.

results in a mixture of particles with genomic DNA or various DNA impurities of different sizes [4–6]. The rAAV genome is designed as the gene of interest (GOI) with two ITRs at both ends. The classical approach to rAAV production relies on helper virus-free transfection methods in mammalian cells using three plasmids, namely a cis-plasmid carrying the GOI and ITRs, a trans-plasmid encoding the adeno-associated virus (AAV) capsid and replication proteins, and a helper plasmid supplying helper virus functions.

The production systems involving mammalian, insect, and yeast cells face significant challenges in generating and removing empty particles lacking complete rAAV. These empty particles constitute over 50% of the total rAAV particles. Incompletely filled and empty particles significantly affect the therapeutic potency and safety of rAAV. Additionally, the presence of impurity DNA within the particles, such as plasmids or host cell DNA, poses further safety concerns. Quantifying, identifying, and characterizing the sequences within these particles, including all the separated subpopulations, has become crucial for understanding particle heterogeneity. High-throughput sequencing (HTS) is a powerful tool for comprehensively characterizing rAAV genomic and impurity DNA [7,8]. Quantitative PCR offers high sensitivity and specificity; however, its efficiency in measuring unexpected DNA is limited [9–11].

In this study, pAAV-ZsGreen1, pRC2-mi342, and pHelper were used to produce rAAV2. Hence, rAAV particles incorporated rAAV genomic DNA and DNA fragments from plasmid and host cell DNA of different lengths. The separation of these particles into subpopulations based on the DNA content allows the characterization of their heterogeneity and variation range. In this study, we investigated the DNA composition of isolated subpopulations of the rAAV particles obtained using anion exchange (AEX) chromatography. Particle DNA comprising rAAV genomic DNA and impurities originating from production plasmids and producer cell DNA were quantified and characterized using droplet digital PCR (ddPCR) and MiSeq. By thoroughly profiling the distribution of genomic DNA and impurities in these subpopulations, this study provides valuable insights into the range of heterogeneity of particle-associated DNAs, offering critical clues regarding the efficiency of the packaging process, which is the key limiting step in rAAV production.

2 | Materials and Methods

2.1 | Fractionation of rAAV2-ZsGreen1 Through AEX Chromatography

Affinity-column-purified rAAV2-ZsGreen1 was kindly provided by the Manufacturing Technology Association of Biologics (MAB). In brief, the rAAV2-ZsGreen1 particle was produced using a three-plasmid transfection method (Figure S1). Two lots of rAAV2-ZsGreen1 were produced using VPC2.0 cells (ThermoFisher Scientific) in 12-L (Lot-K22A16) and 45-L (Lot-K22M5) scale (Figure S2). To prepare the AEX chromatography load, the affinity eluate was diluted 5.4-fold with 25 mM bis-tris-propane (BTP) to adjust the pH to 9.0 and reduce the load conductivity to approximately 3.5 mS/cm. The AEX fraction of the rAAV2-ZsGreen1 particles was measured using an AKTA

avant150 chromatography system (Cytiva) and monitored using inline pH, conductivity, and UV-VIS sensors. rAAV2-ZsGreen1 particles were loaded into a 1-mL CIMmultus QA monolith column (Sartorius BIA Separations). The dual-ion linear gradient elution protocol was as follows: equilibration using 100% Buffer A over 10 column volumes (CVs), sample loading over 100 CVs, column washing using 100% Buffer A over 10 CVs, elution with a linear gradient from 100% Buffer A to 100% Buffer B over 80 CVs, and column cleaning using 100% Buffer A over 10 CVs. The flow rate was maintained at 2 CV/min. Buffer A contained 25 mM BTP and 1 mM MgCl₂ (pH 9.0), and Buffer B contained 25 mM BTP, 10 mM MgCl₂, and 60 mM NaCl (pH 9.0). rAAV2 capsids were collected into 12 fractions (5 mL each) and stored at –80°C.

The rAAV particle protein composition of each AEX fraction was analyzed using sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Subsequently, 10 µL of each fraction was loaded onto the gel and subjected to denaturing protein electrophoresis under standard running conditions. Following electrophoresis, the gel was stained with Instant Blue Coomassie Protein Stain (Abcam) according to the manufacturer's instructions and visualized using ImageQuant LAS 4000 (Cytiva).

2.2 | ddPCR for rAAV2-ZsGreen1, AAV Plasmids, and the 18S rDNA Gene

For ddPCR, DNase treatment and particle DNA extraction were performed. DNase I (Takara Bio) was added to the AEX fraction (1 unit per 20 µL and held at 37°C for 30 min). EDTA (Nacalai Tesque Inc.) was added at a final concentration of 50 mM to stop DNase I digestion. Heat treatment at 95°C for 10 min was conducted to release particle DNA. These samples were diluted with 1 × TE_{PF} (10 mM Tris (hydroxymethyl) aminomethane-HCl buffer, 1 mM EDTA, and pH 8.0 containing 0.01% Pluronic F-68 (Sigma-Aldrich)).

All primers and probes were custom-synthesized and HPLC purified (Eurofins Genomics K. K.) (Table S1). For ddPCR, the reaction mixture comprised 10 µL of ddPCR Supermix (Bio-Rad Laboratories), 2 µL of primers (a final concentration of 0.9 µM each), 1 µL of the probe (final concentration, 0.25 µM), and 1 µL of the sample in a total volume of 20 µL. Droplets were generated in the DG8 cartridge using the QX200 Droplet Generator (Bio-Rad). DNA samples were tested in triplicate at two dilutions. A 96-well plate containing the generated droplets was transferred to a C1000 Touch Thermal Cycler (Bio-Rad Laboratories). The cycling conditions were as follows: 95°C for 10 min, followed by 40 cycles of a two-step thermal profile, comprising 94°C for 30 s and 60°C for 60 s. Subsequently, the plate was transferred to a QX200 droplet reader (Bio-Rad Laboratories), and data analysis was performed using the QuantaSoft software (version 1.7.4.0917; Bio-Rad). The threshold separating the negative and positive droplets was manually set just above the bottom cluster of negative droplets. The concentration of the host cell nuclear DNA was calculated through ddPCR copies [12].

To assess genome completeness, two-dimensional (2D) ddPCR was performed targeting the CMVp region (positions 538–661) and 2389P region (positions 2336–2462) on the 2607-nucleotide (nt) rAAV2-ZsGreen1 genome (Figure S3A). The sequences of the

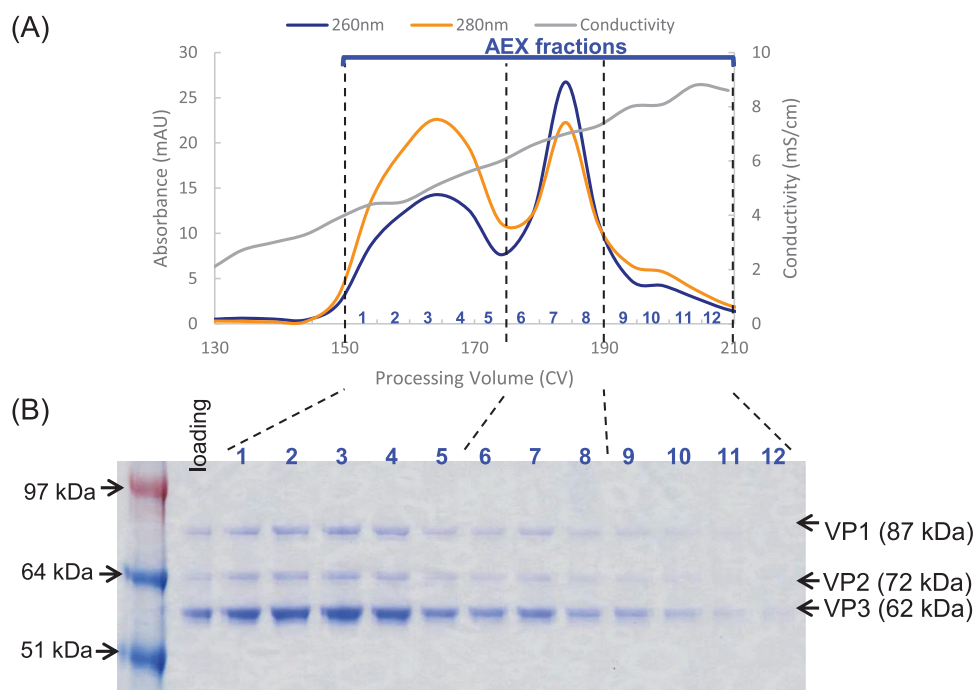


FIGURE 1 | rAAV2-ZsGreen1 fractions separated using anion exchange (AEX) chromatography and analyzed through sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). (A) rAAV2-ZsGreen1 separation layout. rAAV2-ZsGreen1 particles were separated on a 1-mL AEX column, CIMmultus QA, with 5 mL of fractions collected. The elution was performed at pH 9.0 over 120–200 column volume (CV) using a linear gradient of increasing dual-ion strength from 25 mM bis-tris propane (BTP), 1 mM MgCl_2 to 25 mM BTP, 10 mM MgCl_2 , and 100 mM NaCl. Two distinct peaks were observed, namely Fr-1 to Fr-5 in Peak 1 and Fr-6 to Fr-8 in Peak 2. The x-axis represents the retention volume (or the CV). Blue and red traces correspond to absorbance at 260 nm (A_{260}) and 280 nm (A_{280}), respectively, indicating the presence of nucleic acid and protein, respectively. The gray trace corresponds to the solution conductivity, indicating the ionic strength of elution conditions. (B) SDS-PAGE of the collected fractions. SDS-PAGE was performed to assess the presence of rAAV2-ZsGreen1 capsid proteins in the collected fractions. Lane 1: molecular weight markers. Lane 2: AEX loading sample. Lanes 3–14 correspond to 12 fractions collected during AEX chromatography. Visible bands correspond to the rAAV2-ZsGreen1 capsid proteins VP1 (87 kDa), VP2 (72 kDa), and VP3 (62 kDa).

primers and probes used for each target are shown in Figure S3B. The 2D ddPCR was conducted under the same conditions as the singleton ddPCR described in this study. Genome completeness (linkage rate) was defined as the CMVp and 2389P double-positive rate relative to the total counts of CMVp and 2389P amplicon and was calculated using the Poisson-correction formula (Figure S3D).

2.3 | MiSeq Sequence and Bioinformatics Analysis

DNA extraction was performed as previously described, using DNase I to remove particle-free DNA, heat treatment to release particle DNA, and AMPure XP beads (Beckman Coulter) for particle DNA cleanup [13]. Particle DNA extracted from each AEX fraction was finally eluted in 66 μL of ddH_2O (Ambion). Second-strand synthesis was performed in a 100- μL reaction, consisting of a hexamer mix of 2.9×10^{13} oligos (485 nmol/L) (New England BioLabs), 2 mmol/L of each dNTP (New England BioLabs) and 20 units of Klenow fragment (New England Biolabs), and 64 μL of DNA extract, through a ramp of $0.1^\circ\text{C}/\text{s}$ until 37°C , and held at 37°C for 1 h. Sample dsDNA was purified using 1.8 $\times$ AMPure XP beads (Beckman Coulter) and finally eluted in 20 μL of ddH_2O (Ambion Inc.). The dsDNA concentrations were determined using a Qubit4 fluorometer with a Qubit 1X dsDNA High Sensitivity Assay kit (ThermoFisher Scientific).

MiSeq libraries were prepared using the NEBNext Ultra II FS DNA Library Prep Kit for Illumina (New England BioLabs). Similarly, 1 ng of dsDNA was fragmented at 37°C for 15 min and 65°C for 30 min, except for Fr-1, which was fragmented at 37°C for 5 min and 65°C for 30 min. Furthermore, 2.5 μM of NEBNext Adaptor, diluted at 0.6 μM in 10 mM Tris and 10 mM NaCl, from the NEBNext Multiplex Oligos for Illumina, Index Primers Set1 (New England Biolabs), was ligated to the fragmented DNA with 30 μL of NEBNext Ultra II Ligation Master Mix and 1 μL of NEBNext Ligation Enhancer at 20°C for 15 min. Opened dsDNA ends of the fragments were digested with NEBNext Multiplex Oligo USER enzyme at 37°C for 15 min and purified using 0.8X AMPure XP beads (Beckman Coulter). Library PCR was performed as follows: 98°C for 30 s, followed by 9 cycles of a two-step thermal profile, comprising 98°C for 10 s and 65°C for 75 s. Library products were purified using 0.9X AMPure XP beads (Beckman Coulter) and eluted in 22 μL of 0.1X TE buffer. The DNA library size was assessed using a 4150 TapeStation System (Agilent Technologies) with a high-sensitivity D5000 kit. Library concentrations were determined on a Qubit 4 fluorometer using a Qubit 1X dsDNA High Sensitivity Assay kit (Thermo Fisher Scientific). The adapter-ligated DNA fragments were quantified through real-time PCR using the NEBNext Library Quant Kit for Illumina (New England Biolabs). PhiX Control v3 (Illumina) was added to the pooled libraries, which were sequenced using MiSeq (Illumina). MiSeq reads were 300 bp $\times$ 2 in length with

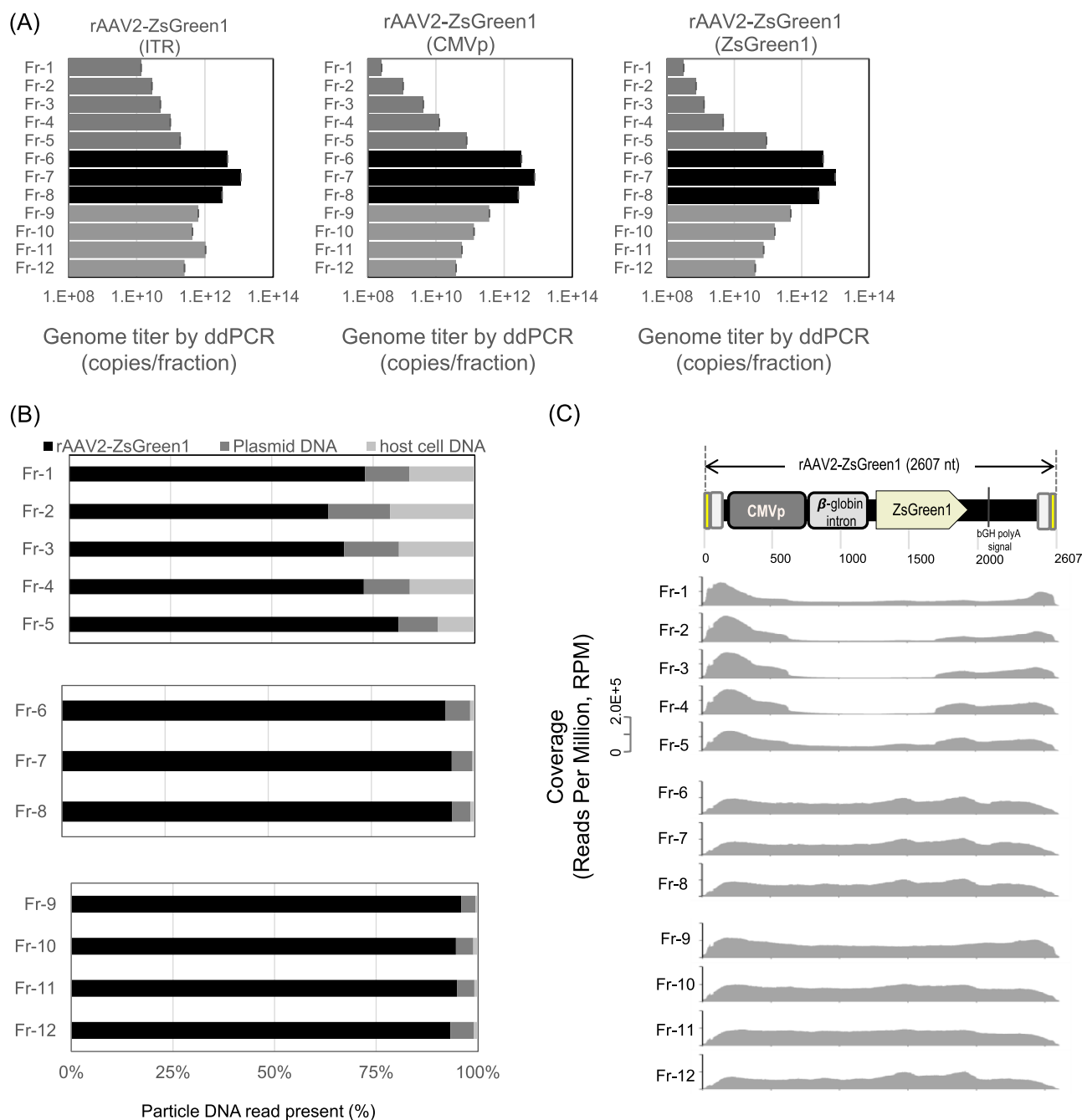


FIGURE 2 | rAAV2-ZsGreen1 particle genomic DNA analyzed using droplet digital PCR (ddPCR) and MiSeq. (A) ddPCR quantification of particle genomic DNA. The particle genomic DNA across AEX fractions was quantified using ddPCR through the estimation of three regions on rAAV2-ZsGreen1 genomes, involving ITR, CMVp, and ZsGreen1, shown in Figure S1. The error bars represent the range of ± 3 standard deviations (σ) for a sample size of $N = 3$. (B) MiSeq analysis of the frequency of the rAAV2-ZsGreen1 genome from AEX fractions. The percentages of MiSeq reads are shown. rAAV2-ZsGreen1 genomic DNA proportion is denoted using the black bar, plasmid DNA is denoted in dark gray, and host cell DNA in light gray. (C) Mapping profiles of the rAAV2-ZsGreen1 genome. Mapping profiles were generated by aligning the MiSeq reads against the reference sequence, rAAV2-ZsGreen1 genomic DNA (2607 nt in length). rAAV2-ZsGreen1 genomic structures are illustrated at the top. The y-axis is presented in a linear scale.

a single index. Read trimming was performed using fastp v0.22.0 [14] with parameters “-5 -3 -y -q 30 -l 40.” Sequential alignments of trimmed reads were performed against rAAV2-ZsGreen1, pAAV-ZsGreen1 backbone, pRC2-mi342 plasmid, pHelper plasmid, and GRCh38.p14 (GCF_000001405.40), using HISAT2 v2.1.1 [15] with the default parameters. Aligned reads were normalized to reads

per million (RPM) to facilitate comparison amongst samples. Coverage depths were calculated using SAMtools v1.15.1 [16] with the command “depth -a.” The depth at each position of the reference sequence was used to generate a mapping profile using a customized Python script implemented with Biopython.

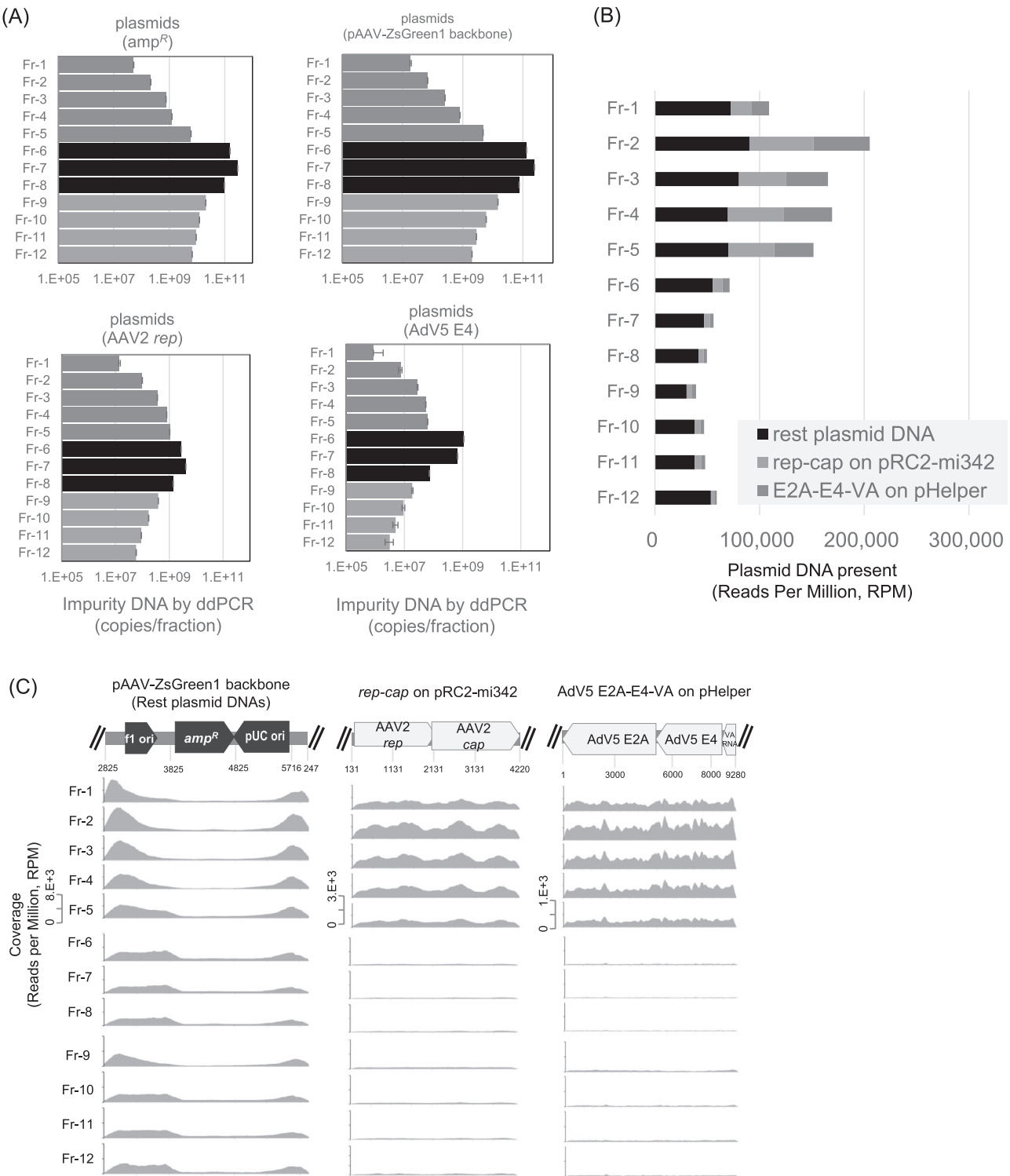


FIGURE 3 | Analysis of plasmid impurities using ddPCR and MiSeq. (A) Plasmid DNA measurement using ddPCR. The target amplicons were *amp^R* on three plasmids, a unique region on pAAV-ZsGreen1 backbone (3641–3737), AAV2 *rep* on pRC2-mi342 (843–918), and AdV5 E4 on pHelper (7110–7255). The error bars represent the range of ± 3 standard deviations (σ) for a sample size of $N = 3$. (B) MiSeq reads originated from three plasmids across AEX fractions. The number of reads mapped to each reference was counted and normalized to 1,000,000 reads in total. (C) Mapping profile on plasmid DNAs. The y-axis represents the linear range. Three reference plasmid sequences were as follows: pAAV-ZsGreen1 backbone, 2825–5716 and 1–247 (representing the plasmid backbone sequences on pRC2-mi342 and pHelper), rep and cap genes on pRC2-mi342 (131–4220), and AdV5 E2-E4-VA RNA genes on pHelper (1–9280).

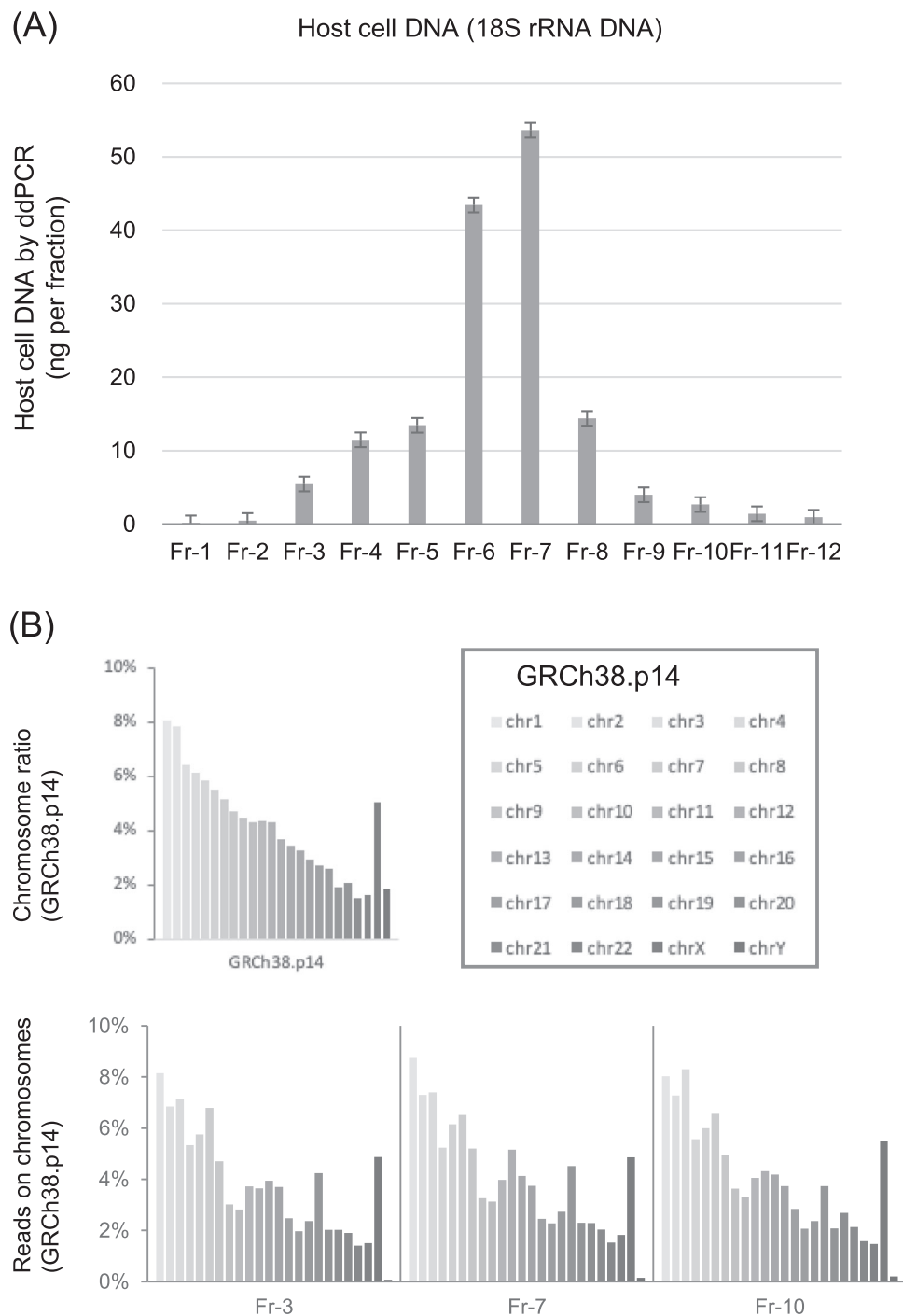


FIGURE 4 | Host cell DNA quantification through ddPCR and the analysis of its origin from the human genome using MiSeq. (A) Quantification of encapsulated host cell DNA was determined across AEX fractions using ddPCR. ddPCR was performed targeting 18S rDNA (18S-F2), a representative of human genomes. Subsequently, the host cell DNA mass (ng) was estimated based on 18S rDNA copies. The y-axis represents DNA mass (ng). The error bars represent the range of ± 3 standard deviations (σ) for a sample size of $N = 3$. (B) Distribution of host cell DNA reads on chromosomes. MiSeq reads aligned against the human genome (GRCh38.p14). Host cell DNA reads were collected and clustered into each chromosome, through chr1 to chr22, chrX, and chrY. The percentages of total reads for each chromosome represent the origin chromosomes of host cell DNA impurities packed in AEX-fractionated particles. For standardization, the left panel shows the size percentages of chromosomes chr1 to chr22 and chrX and chrY in the human genome. The distribution of reads from Fr-3, Fr-7, and Fr-10 particle DNA is displayed. The y-axis shows the percentages.

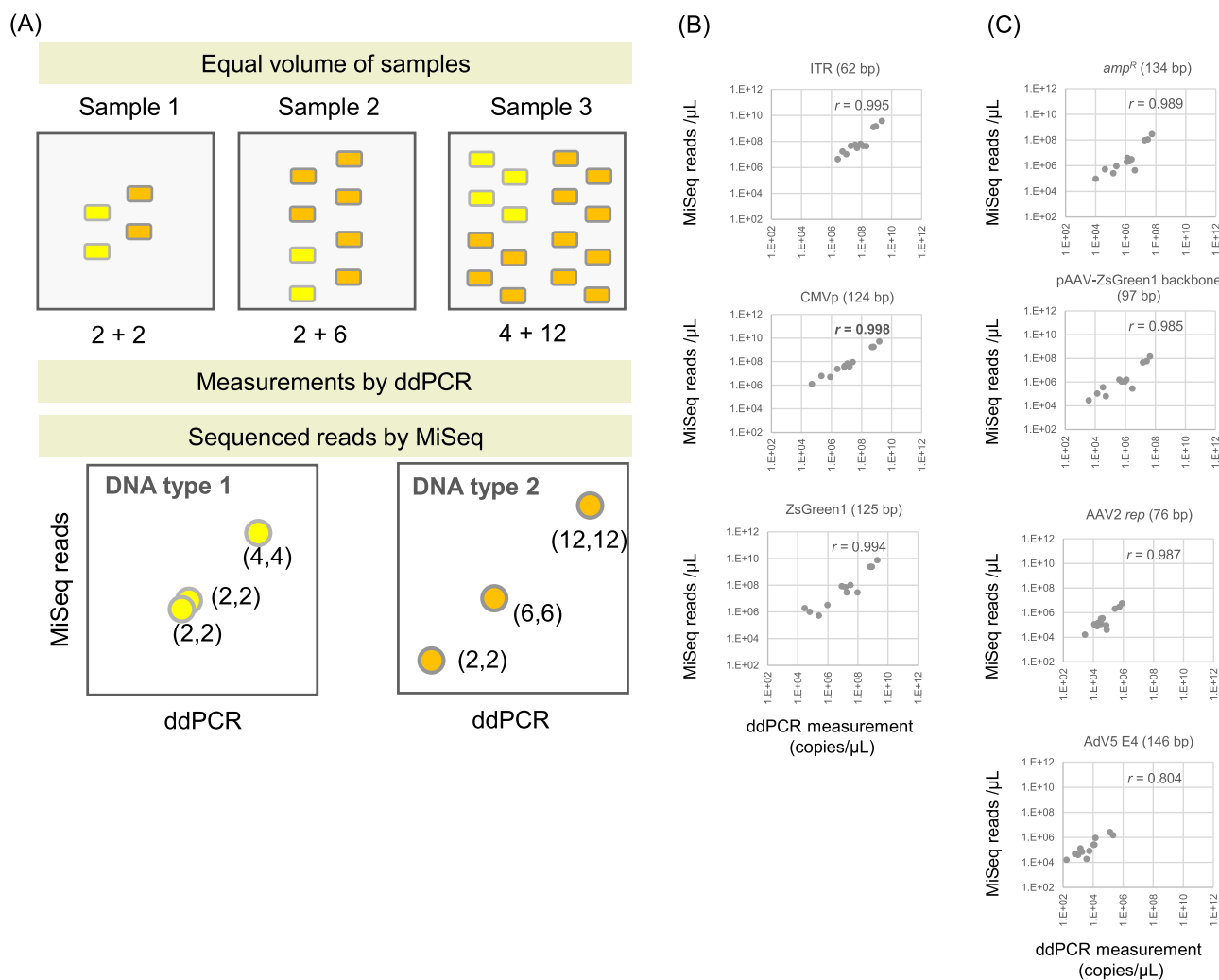


FIGURE 5 | Quantitative correlation of ddPCR and MiSeq. (A) Workflow illustration of MiSeq quantitative analysis among variable rAAV samples. Sample DNA was extracted and sequenced using MiSeq. Following the MiSeq procedures, the original DNA from variable samples was proportionally augmented through second-strand synthesis and library PCR. Read counts were normalized to original sample volumes, resulting in read counts per sample volume (1 μ L). Normalized reads in ddPCR amplicon regions were counted. Ideally, MiSeq provides quantitative sequencing data, while ddPCR enables absolute quantification. rAAV particles, including empty and full particle containing the variable genome/impurity ratios. Two types of DNA molecules represent impurity DNA (type 1) and rAAV genome (type 2). The relative abundance of these DNA types shifts in a gradient manner across empty to full particles. Plotting MiSeq and ddPCR values across samples reflects the quantitative capability of both methods. (B) Correlations of ddPCR and MiSeq analysis targeting regions on rAAV2-ZsGreen1. The regions analyzed included the ITR (62 bp at positions 83–144 and 2464–2525), CMVp (124 bp at 538–661), and ZsGreen1 (125 bp at 1345–1469). (C) Correlations of ddPCR and MiSeq analyses targeting regions within impurity DNAs. This analysis focuses on *amp^R* in three plasmids, pAAV-ZsGreen1 backbone, AAV2 rep, and AdV5 E4. For each targeting region, ddPCR measurements were plotted on the x-axis, and MiSeq reads were plotted on the y-axis. In both axes, log10 transformation of original count number is used. Across 12 AEX fractions, DNA amount was low in empty particle and high in full particles. Although 1 ng of DNA was used for the sequencing library preparation. MiSeq read counts were normalized to a 1 μ L equivalent of the original sample. This normalization allows for direct comparison of read abundance across samples with the variable concentration. Pearson correlation analysis was used to evaluate the positive correlation between MiSeq read counts and ddPCR values. Pearson correlation coefficient (r) falls in 0.9–1.0, supporting the strong positive correlation and revealing MiSeq workflow robustness.

3 | Results and Discussion

3.1 | Fractionation of rAAV2-ZsGreen1 Particles Using AEX Chromatography

A fundamental concern in rAAV production is the yield-associated rAAV particle heterogeneity. In this study, particle heterogeneity was examined by analyzing the subpopulations of particles with varying masses. This was accomplished by fractionating particles into subpopulations based on their mass

and analyzing the distribution of the particle DNAs. Fractionation was performed using AEX chromatography, a method for separating particles based on their charge, which correlates with their DNA content and mass. Two rAAV2-ZsGreen1 lots (Lot-K22A16: $3.0\text{E}+13$ genomes, $3.5\text{E}+14$ capsids; Lot-K22M5: $1.6\text{E}+13$ genomes, $4.7\text{E}+13$ capsids) were separated on a 1-mL column of CIMmultus QA using continuous-gradient elution. A subpopulation of rAAV particles was collected after the AEX column (5 mL for each fraction, Fr-1 to Fr-12) based on their net negative charges and aggregated sizes. Two peaks were observed:

Fr-1 to Fr-5 in Peak 1 and Fr-6 to Fr-8 in Peak 2 (Figures 1A and S2). The shoulder on the right side of Peak 2, Fr-9 to Fr-12, was considered to be a debris aggregate [17] or overloaded particles [18]. By estimating the absorbance ratio at 260 and 280 nm inline during AEX separation, Peak 1 was determined to be empty particles with an $A_{260/280}$ ratio close to 0.7 at the peak top, and Peak 2 was identified as full particles with a 1.3 of the $A_{260/280}$ ratio at the peak top [19, 20]. Distinct peaks indicate the elution of rAAV2 particles in different fractions, suggesting variability in particle forms or states. SDS-PAGE confirmed the presence of the rAAV2 capsid, VP1, VP2, and VP3 in Fr-1 to Fr-12 (Figures 1B and S2). Combined analysis of AEX elution and SDS-PAGE indicated the distribution and purity of rAAV2 particles across the fractions, revealing the efficiency of the purification process and the quality of the isolated particles. Reproducible AEX retention profiles were observed for two rAAV2 lots. Particle DNA analysis was conducted on fractions from 12-L Lot-K22A16 and 45-L Lot-K22M5 (data in Supporting Information).

Empty particles present a major challenge in rAAV production. The empty particles constituted approximately 73% of the total particle population. This rate of empty particles (approximately 80%–90%) was also observed in a previous study [21]. This suggests a limited supply of rAAV genomic DNA relative to the capsid shells available in our production system. Dynamic observations of genomic packaging confirmed this limited supply in insect cells. Within these empty particles, the presence ratio of DNA fragments was less than one DNA fragment per 100 particles (approximately) in this study. Unlike Troxell et al., [22], almost 90% of the particles in the light fractions carried varying amounts of DNA, as demonstrated through CD-MS, suggesting that the primary limitation is the availability of complete genomic DNA. rAAV particles are produced in a sequential manner, forming empty-capsid shells and then forcing ITR-containing DNA into empty shells. The formation of empty and incomplete genomic particles is closely related to DNA packaging. Empty particles are formed owing to the lack of DNA, which is limited by the DNA replication rate. Incomplete genomic particles are formed by packaging events involving ITR DNA that lacks complete elongation. Increasing the rate of DNA replication could enhance the packaging efficiency and reduce the proportion of empty particles. However, other factors such as capsid stability, cellular environment, and competition for packaging factors may also influence this process. Understanding the mechanisms underlying empty particle formation is essential for developing strategies to improve the yield and purity of rAAV. Higher titers of complete rAAV vectors can be achieved by addressing the limitations in DNA replication and packaging. To improve the purification of the pooled particles, recycled N-rich AEX separation was performed, and the genomic homogeneity was increased, addressing one solution of purity insurance [21].

3.2 | rAAV2-ZsGreen1 Genomic DNA Fluctuations in AEX-Separated Particle Subpopulations

To determine the distribution of genomic DNA across the AEX fractions, the rAAV genomic DNA content was determined through ddPCR, targeting three locations on the rAAV2-ZsGreen1 genome, ITR, CMVp, and ZsGreen1 (Figures 2A and S1, Table S2). In 12-L Lot-K22A16 AEX fractions, ITR measurements showed

an increase, ranging from $1.3\text{E}+10$ in Fr-1 to $1.1\text{E}+13$ in Fr-7 at the highest value and a decline to $2.5\text{E}+11$ copies in Fr-12. CMVp measurements showed the same pattern, an increase from $2.5\text{E}+8$ to $7.8\text{E}+12$ and a decrease to $3.9\text{E}+10$ copies. The ZsGreen1 measurement showed an increase from $3.1\text{E}+8$ to $9.9\text{E}+12$ and a decrease to $4.2\text{E}+10$ copies per fraction. Based on ion exchange separation, the DNA content in purified particle subpopulations presents a trend ranging from a few amounts of DNA to a complete genome, shown as approximately equal amounts of ITR, CMVp, and ZsGreen1. Within Peak 1, the particle DNA was expected to consist of fragmented DNA; that is, small-sized incorporated DNA. From Fr-1 to Fr-4, the ITR values were 42.7–20.0 times higher than those of ZsGreen1, showing a higher existing frequency of ITR fragments in particles than the fragments of the CMVp and ZsGreen1 sequences, indicating that ITR was preferentially packed into particles, agreeing with ITR function as a packaging element [23]. This result highlights the ITR measurement bias when handling samples with unknown DNA composition, particularly rAAV particle mixtures. The ZsGreen1 measurement was used to describe the results in the subsequent sections of this study.

Additionally, genome integrity was assessed using duplex two-dimensional (2D) ddPCR [24, 25], targeting the upstream CMVp promoter region and the downstream 2389P region on the 2607-nt genome (Figure S3A). These two amplicons span a 1925-nt region, covering the gene expression cassette. AEX fraction Fr-3 (empty particle) and Fr-7 (full particle) exhibited 20.7% and 74.21%, respectively, of the CMVp-2389P fragment among all genomic molecules detected. This confirms the effectiveness of AEX chromatography in separating viral particles and a strong tendency of intact genome incorporation in full particles. In the empty-particle Peak 1, the ITR signals accounted for almost 2% of all ITR signals across all AEX fractions. This indicates that ~2% of DNA-positive capsids were found within the empty particle subpopulation. The 20.7% linkage in Peak 1 likely originates from a minor population of full particles remaining in empty particle Peak 1 after AEX purification.

Further, DNA compositions were determined using MiSeq, and the heterogeneity of the rAAV particles was thoroughly screened. Reads aligned on rAAV2-ZsGreen1, plasmids, and host genomes were clustered, and the read percentages are presented in Figure 2B. From Fr-1 to Fr-12, the ratio of rAAV2-ZsGreen1 reads was increased from 73.0% to 95.9% (Figure 2B, Table S3), indicating an increased purity with increasing DNA content in the particle subpopulations. The mapping of the rAAV2-ZsGreen1 genome provides detailed insights into the distribution and integrity of genomic DNA across the AEX fractions. In agreement with ddPCR, the ITR region with higher abundance revealed particle DNA incorporation relative to the presence of the ITR (Figure 2C). For Lot-K22M5, DNA components in fractions Fr-3 (empty), Fr-7 (full), and Fr-10 (post-full) were analyzed using ddPCR and MiSeq (Tables S2 and S3). ddPCR revealed ITR/ZsGreen1 ratios of 16.3, 1.0, and 1.1 in Fr-3, Fr-7, and Fr-10, respectively. MiSeq analysis showed that Fr-3, Fr-7, and Fr-10 contained 69.95%, 93.21%, and 93.06% rAAV genomic DNA, respectively (Table S3), and their mapping profile is shown in Figure S5. Consistent with Lot-K22A16, the 45-L scale Lot-K22M5 also exhibited an enrichment of ITRs in empty particles and complete genomes in full particle fractions.

The rAAV particles carry DNA fragments of varying sizes, we observed such variation in the sizes of the incorporated rAAV genomes. Particle DNA across fractions showed different frequencies within genomic DNA regions, with the ITR having the highest prevalence across all fractions. This elevated ITR frequency supports the idea that ITR initiates packaging [23, 26]. This result is consistent with that of Tran, who reported the highest frequency of ITR presented within the heavy, moderate, and light fractions purified by CsCl ultracentrifugation [7]. Furthermore, particle DNA length within heavy, moderate, and light CsCl fractions was estimated by charge detection mass spectrometry (CD-MS) [18], showing that the incomplete rAAV genomes greatly impacted GOI expression, as revealed by a cell-based assay [22]. This underscores the significance of ensuring complete genome packaging and achieving high purity of full-filled particles with a homologous complete genome to maintain their functionality and efficacy in gene therapy.

3.3 | Plasmid Impurities Across AEX-Fractionated rAAV2-ZsGreen1 Particle Subpopulations

The DNA components of the plasmid impurities were analyzed within the particle subpopulations after AEX fractionation. Three production plasmids were quantified using ddPCR, targeting representative amplicons (*amp^R*), with the *amp^R* test for the sum of the three plasmids: the pAAV-ZsGreen1 amplicon at its unique region on its plasmid backbone, the AAV2 rep gene on pRC2-mi342, and the AdV5 E4 within AdV5 E2A-E4-VA RNA sequences on pHelper (Figure S1). These *amp^R* sequences are used to evaluate the risk associated with rAAV particles due to the presence of the antibiotic resistance gene. Similar to the rAAV genomic DNA distribution across rAAV2-ZsGreen1 (12-L Lot-K22A16) AEX fractions, all plasmid measurements showed increased values from Fr-1 to Fr-7, which declined until Fr-12 (Figure 3A), consistent with the theoretical DNA content distribution across AEX fractions. The largest increase was observed in *amp^R* ddPCR measurements among the AVB Sepharose's AEX fractions, reaching 5600 times from 5.2E+7 to 2.9E+11 copies in Fr-1 to Fr-7 (Table S2). Comparison of the top values of 2.9E+11 for *amp^R*, 2.4E+11 for the pAAV-ZsGreen1 backbone, 4.1E+9 for AAV2 rep, and 6.7E+8 for AdV5 E4 in Fr-7 (Table S2), indicated that among plasmid-origin impurities, the pAAV-ZsGreen1 origin constituted 85.6% in Fr-7, and 78.1% and 83.4% in Fr-6 and Fr-8, respectively. These results show that approximately 80% of the plasmid DNA was of pAAV-ZsGreen1 origin in full particles. Furthermore, the ratio of *amp^R* to ZsGreen1 (Table S2) represents the plasmid impurities in the particle DNAs. For full particle collection, 3.6% (Fr-6), 2.9% (Fr-7), and 3.0% (Fr-8) were used to estimate the plasmid percentages in the particle DNAs.

Furthermore, 7.3%–11.0% of plasmid DNA was present in empty particle collections, while 6.0% (Fr-6), 5.0% (Fr-7), and 4.5% (Fr-8) of plasmid DNA was found in full particle collections, as determined using MiSeq (Figure 3B, Table S3). The reads were normalized to reads per million, and the three plasmid-origin reads are shown in Figure 3B. For empty particle collections, including Fr-1 to Fr-5, plasmid DNA comprised 1.5%–3.3% of AAV2 rep-cap reads, 1.3%–2.9% of AdV5 E2A-E4-VA reads, and

7.0%–9.0% of the remaining plasmid DNAs. However, for full particle collections, including Fr-6, Fr-7, and Fr-8, plasmid DNA comprised 5.5%, 4.7%, and 4.2% plasmid backbone sequences, respectively, and less than 0.3% of others (Table S4). Consistent with ddPCR, plasmid backbones, possibly the pAAV-ZsGreen1 backbone, were the major origin of plasmid impurities in the full particle collections. Normalized read counting was used to generate mapping profiles (Figure 3C). Within the full particle collection (Fr-6 to Fr-8), the relatively high abundance of pAAV-ZsGreen1 sequences, compared with those of AAV2 rep-cap and AdV5 E2A-E4-VA, indicates that most of the impurities originated from the pAAV-ZsGreen1 backbone, other than two plasmids providing those virus gene sequences, AAV2 rep-cap and AdV5 E2-E4. To estimate the origin of plasmid impurities, the mapping profile of three production plasmids was determined (Figures S4A, S5A, Fr-3, Fr-7, and Fr-10). To investigate ITR-driven packaging, reads were aligned on pAAV-ZsGreen1 only, revealing reads that crossed the ITR region. The fragments encompassing the ITR and pAAV-ZsGreen1 backbone are illustrated in Figures S4B and S5B. The cis-plasmid (pAAV-ZsGreen1) and the rAAV genome share a 2577 bp overlap, while the rAAV genome is 2607-nt long, with a 15 nt ITR A-region complementing the rAAV genome to form the intact AAV2 ITR. Read abundance near the ITR region was higher in empty particles, while no preferential pattern was observed in full particles, suggesting a relatively high purity of the full particle collections.

DNA impurities in the second lot of rAAV2 particles (Lot-K22M5), fractions Fr-3, Fr-7, and Fr-10, were determined by ddPCR and MiSeq. Impurity levels, evaluated as the ratio of *amp^R*/ZsGreen1, were 17.55%, 5.02%, and 4.64% by ddPCR (Table S2) and 10.0%, 6.2%, and 6.1% by MiSeq (Table S3). These results are coincidence with the DNA impurity levels observed in Lot-K22A16 AEX fractions. Two lots of particles run on AEX column with loading amounts of 3.5E+14 and 4.7E +13 capsids and exhibited the identical purification efficiencies, demonstrating the reproducibility of rAAV production.

These results showed that the full particles were still mixed. The impurities in the full particles primarily originated from the cis-plasmid, pAAV-ZsGreen1. Impurities, including residual plasmid and host cell DNA, may generate the un-intended gene expression and random genomic integration. Additionally, the over-presented fragment DNA has potential risk arising the un-noticeable immune response. The presence of impurity DNA transcripts in recipient cells in vivo [2] was detected through RNA-seq even 8 weeks after administration [27]. Several strategies have been employed to address risk concerns and reduce plasmid impurities inherent in production. One approach is to limit plasmid incorporation into particles by using a larger plasmid backbone that exceeds the packaging capacity. Another strategy involves the removal of the cis-plasmid backbone during the *E.coli* cultivation stage, referred to as the linear covalently closed (LCC) plasmid strategy. Additionally, the low-cis transfection strategy significantly reduces cis-plasmid incorporation by decreasing the amount of input cis-plasmids to a 1:100 ratio by weight compared with rep-cap and helper plasmids [27]. By optimizing the plasmid design and transfection protocol, these strategies enhance the purity of fully filled particles.

3.4 | Description of Host Cell DNA

To assess the presence of host cell DNA, 18S rDNA was quantified using ddPCR (Figure 4A). The mass of host cell DNA was calculated by converting the 18S rDNA copies to DNA mass. Based on the ratio of 18S rDNA 5.6×10^4 copies on 1 ng of HEK293 genome [12]. Host DNA exhibited a similar pattern to plasmid impurities across the AEX fractions, increasing from 3.9×10^4 to 1.3×10^7 copies in Fr-1 to Fr-7 and decreasing to 2.3×10^5 in Fr-12 (Table S2, Lot-K22A16). In our full particle fractions (Fr-6 to Fr-8), the estimated host cell DNA levels were 9.57, 4.77, and 4.55 pg per 1.0×10^9 genomes, respectively.

For MiSeq data, investigating host cell DNA impurities originating from human chromosomes was performed. The reads were clustered according to their chromosomal origin. The distribution of host cell DNA reads across the chromosomes was plotted as percentages (Figure 4B). The read percentages across the AEX-fractionated particles showed a distribution pattern where more reads were aligned on longer chromosomes (Figures 4B and S6), indicating that the host cell DNA equivalently originated from the host genome. Homologous sequences of RBEs distributed on chromosomes were found after AAV DNA integration into host cell genomes [4, 28], and these integration sites were identified as hotspots. Encapsulation of host cell DNA into particles is likely initiated by the Rep protein binding to host cell DNA, preferentially at these hotspots, resulting in the inclusion of host cell DNA into the particles.

Guidelines recommend limiting free residual host DNA to <10 ng per dose and <200 bp in size. This is challenging for rAAV production due to the potential for host DNA to be inadvertently packaged within the viral capsids. Considering typical dose ranges of 1.0×10^{13} to 1.0×10^{14} vg/kg, and the presence of other residual impurity DNA (including plasmid DNA), effective purification is crucial. AEX separation addresses this need by enriching for rAAV particle fractions with the highest DNA content, thereby reducing overall impurity levels.

3.5 | MiSeq Quantitative Assessment

When applying MiSeq analysis to rAAV samples, a quantitative assessment of MiSeq data was also performed among samples, using ddPCR measurements as comparable standards. Hence, by targeting the ddPCR amplicons, MiSeq reads within these regions were extracted and counted. For comparison of the two analytical methods, ddPCR and MiSeq, the copy numbers measured using ddPCR and MiSeq reads in amplicons were normalized to a test volume of 1 μ L equivalent to the original sample (Figure 5A). Amplicons related to rAAV2-ZsGreen1 (e.g., ITR, CMVp, and ZsGreen1) and plasmid impurities (e.g., *amp^R*, pAAV-ZsGreen1 backbone, AAV2 rep, and AdV5 E4) were plotted as ddPCR measurements along the x-axis and as MiSeq reads along the y-axis (Figure 5B,C). Since values span several orders of magnitude, a log₁₀ transformation was applied, and the covariance was evaluated between MiSeq and ddPCR measurements. For ITR, CMVp, and ZsGreen1 across the AEX fractions (Lot-K22A16), Pearson correlation coefficients (*r*) were 0.995, 0.998, and 0.994, respectively. These were 0.989, 0.985, 0.987, and 0.804 for the plasmid impurities *amp^R*, pAAV-ZsGreen1 backbone, AAV2 rep,

and AdV5 E4, respectively. These strong correlations across the 12 AEX samples suggest that MiSeq data accurately reflects the molecular proportions in the original samples. In these 12 fractions, both methods yielded comparable data on each target. By comparing the agreement between MiSeq reads and ddPCR values, the consistency of MiSeq performance throughout sequencing runs was confirmed (Figure 5B,C). Given the long MiSeq workflow, through DNA extract and second-strand synthesis to fragmentation and library preparation, and ultimately sequencing on the flowcell, the systematic bias inherent to the MiSeq platform was observed [29, 30]. To mitigate this bias and enable reproducible relative quantitation, the incorporation of internal and processing controls at well-defined concentrations is crucial. This approach would make MiSeq suitable for analyzing rAAV genomes, which are characterized by low complexity and the presence of a small amount of impurities.

The target quantification method, ddPCR, has significant limitations in handling unexpected DNAs. In the HTS screening of the full spectrum of DNA molecules in the samples, the abundance of each DNA component presented the physical ratio of molecules in the samples, theoretically in an unbiased manner. In addition, by quantifying the total HTS dataset, each DNA composition in this dataset could be quantified subsequently, offering a relatively quantitative test.

4 | Conclusion

In this study, a detailed assessment of the heterogeneity of the rAAV particle populations was conducted after rAAV2-ZsGreen1 was fractionated into AEX subpopulations. Across the AEX fractions, the amount of encapsulated DNA increased in agreement with the increased net charge of the particles. Regarding the increased DNA content, rAAV genomic completeness was increased, and impurity DNA was reduced, revealing the spectrum of genomic and impurity DNA within the AEX-fractionated subpopulations. Based on these observations, three notable particle purity concerns were identified: plasmid-origin impurity DNA, rAAV genome length variance, and empty particles. From a pharmaceutical perspective, these impurities raise concerns about their potential risks and the challenges in effectively removing them from rAAV preparations. Ensuring batch-to-batch consistency and minimizing impurities is critical for therapeutic efficacy and regulatory compliance.

Author Contributions

Noriko Hashiba conducted the experiments, performed the data analysis, and drafted the manuscript. Emi Itou-Kudo joined data analysis. Kazuhisa Uchida, Yuzhe Yuan, Yokoko Masumi-Koizumi, and Keisuke Yusa reviewed the manuscript.

Acknowledgments

K.U. is supported by grant from the Japan Agency for Medical Research and Development (AMED; JP18ae0201001). We appreciate the Manufacturing Technology Association of Biologics (MAB).

Conflicts of Interest

The authors declare no conflicts of interest.

Data Availability Statement

The detailed data and protocols of this study are available upon reasonable request from the corresponding author. MiSeq data were submitted to the NCBI Sequence Read Archive (SRA) with BioProject ID: PRJNA1162916.

References

1. D. H. Bryant, A. Bashir, S. Sinai, et al., "Deep Diversification of an AAV Capsid Protein by Machine Learning," *Nature Biotechnology* 39, no. 6 (2021): 691–696, <https://doi.org/10.1038/s41587-020-00793-4>.
2. G. Chadeuf, C. Ciron, P. Moullier, and A. Salvetti, "Evidence for Encapsulation of Prokaryotic Sequences During Recombinant Adeno-Associated Virus Production and Their in Vivo Persistence After Vector Delivery," *Molecular Therapy* 12, no. 4 (2005): 744–753, <https://doi.org/10.1016/j.ymthe.2005.06.003>.
3. K. Gao, M. Li, L. Zhong, et al., "Empty Virions in AAV8 Vector Preparations Reduce Transduction Efficiency and May Cause Total Viral Particle Dose-Limiting Side Effects," *Molecular Therapy—Methods & Clinical Development* 1, no. 9 (2014): 20139, <https://doi.org/10.1038/mtm.2013.9>.
4. P. Tai, J. Xie, K. Fong, et al., "Adeno-Associated Virus Genome Population Sequencing Achieves Full Vector Genome Resolution and Reveals Human-Vector Chimeras," *Molecular Therapy—Methods & Clinical Development* 9 (2018): 130–141, <https://doi.org/10.1016/j.omtm.2018.02.002>.
5. L. F. Barnes, B. E. Draper, Y. T. Chen, T. W. Powers, and M. F. Jarrold, "Quantitative Analysis of Genome Packaging in Recombinant AAV Vectors by Charge Detection Mass Spectrometry," *Molecular Therapy—Methods & Clinical Development* 23 (2021): 87–97, <https://doi.org/10.1016/j.omtm.2021.08.002>.
6. S. Namkung, N. T. Tran, S. Manokaran, et al., "Direct ITR-to-ITR Nanopore Sequencing of AAV Vector Genomes," *Human Gene Therapy Methods* 33, no. 21–22 (2022): 1187–1196, <https://doi.org/10.1089/hum.2022.143>.
7. N. T. Tran, E. Lecomte, S. Saleun, et al., "Human and Insect Cell-Produced Recombinant Adeno-Associated Viruses Show Differences in Genome Heterogeneity," *Human Gene Therapy* 33, no. 7–8 (2022): 371–388, <https://doi.org/10.1089/hum.2022.050>.
8. E. Lecomte, B. Tournaire, B. Cogné, et al., "Advanced Characterization of DNA Molecules in rAAV Vector Preparations by Single-Stranded Virus Next-Generation Sequencing," *Molecular Therapy—Nucleic Acids* 4, no. 10 (2015): 260, <https://doi.org/10.1038/mtna.2015.32>.
9. G. Wang, Q. Ma, C. Wei, et al., "Assessment of Key Factors Impacting Variability in AAV Vector Genome Titration by Digital PCR," *International Journal of Molecular Sciences* 25, no. 10 (2024): 5149, <https://doi.org/10.3390/ijms25105149>.
10. Y. Wang, N. Menon, S. Shen, M. Feschenko, and S. Bergelson, "A qPCR Method for AAV Genome Titer With ddPCR-Level of Accuracy and Precision," *Molecular Therapy—Methods & Clinical Development* 19 (2020): 341–346, <https://doi.org/10.1016/j.omtm.2020.09.017>.
11. F. Wang, X. Cui, M. Wang, W. Xiao, and R. Xu, "A Reliable and Feasible qPCR Strategy for Titrating AAV Vectors," *Medical Science Monitor Basic Research* 19 (2013): 187–193, [10.12659/MSMBR.883968](https://doi.org/10.12659/MSMBR.883968).
12. K. Higashiyama, Y. Yuan, N. Hashiba, K. Masumi-Koizumi, K. Yusa, and K. Uchida, "Quantitation of Residual Host Cell DNA in Recombinant Adeno-Associated Virus Using Droplet Digital Polymerase Chain Reaction," *Human Gene Therapy Methods* 34, no. 11–12 (2023): 578–585, <https://doi.org/10.1089/hum.2023.006>.
13. Y. Yuan, K. Higashiyama, N. Hashiba, K. Masumi-Koizumi, K. Yusa, and K. Uchida, "Concise Analysis of Single-Stranded DNA of Recombinant Adeno-Associated Virus by Automated Electrophoresis System," *Human Gene Therapy Methods* 35, no. 3–4 (2024): 104–113, <https://doi.org/10.1089/hum.2023.148>.
14. S. Chen, Y. Zhou, Y. Chen, and J. Gu, "fastp: An Ultra-Fast All-in-One FASTQ Preprocessor," *Bioinformatics* 34, no. 17 (2018): i884–i890, <https://doi.org/10.1093/bioinformatics/bty560>.
15. D. Kim, J. M. Paggi, C. Park, C. Bennett, and S. L. Salzberg, "Graph-Based Genome Alignment and Genotyping With HISAT2 and HISAT-Genotype," *Nature Biotechnology* 37, no. 8 (2019): 907–915, <https://doi.org/10.1038/s41587-019-0201-4>.
16. H. Li, B. Handsaker, A. Wysoker, et al., 1000 Genome Project Data Processing Subgroup., "The Sequence Alignment/Map Format and SAMtools," *Bioinformatics* 25, no. 16 (2009): 2078–2079, <https://doi.org/10.1093/bioinformatics/btp352>.
17. D. P. Chen, J. Y. Wei, J. C. Warren, and C. Huang, "Tuning Mobile Phase Properties to Improve Empty and Full Particle Separation in Adeno-Associated Virus Productions by Anion Exchange Chromatography," *Biotechnology Journal* 19, no. 1 (2024): 2300063, <https://doi.org/10.1002/biot.202300063>.
18. T. P. Wörner, J. Snijder, O. Friese, T. Powers, and A. J. R. Heck, "Assessment of Genome Packaging in AAVs Using Orbitrap-Based Charge-Detection Mass Spectrometry," *Molecular Therapy—Methods & Clinical Development* 24 (2022): 40–47, <https://doi.org/10.1016/j.omtm.2021.11.013>.
19. A. K. Werle, T. W. Powers, J. F. Zobel, et al., "Comparison of Analytical Techniques to Quantitate the Capsid Content of Adeno-Associated Viral Vectors," *Molecular Therapy—Methods & Clinical Development* 23 (2021): 254–262, <https://doi.org/10.1016/j.omtm.2021.08.009>.
20. P. R. H. Joshi, A. Bernier, P. D. Moço, J. Schrag, P. S. Chahal, and A. Kamen, "Development of a Scalable and Robust AEX Method for Enriched rAAV Preparations in Genome-Containing VCs of Serotypes 5, 6, 8, and 9," *Molecular Therapy—Methods & Clinical Development* 21 (2021): 341–356, <https://doi.org/10.1016/j.omtm.2021.03.016>.
21. S. Suk Lee, J. Lee, K. Fang, et al., "Separation of Full, Empty, and Partial Adeno-Associated Virus Capsids via Anion-Exchange Chromatography With Continuous Recycling and Accumulation," *Journal of Chromatography B* 1242 (2024): 124206, <https://doi.org/10.1016/j.jchromb.2024.124206>.
22. B. Troxell, S. L. Jaslow, I. W. Tsai, et al., "Partial Genome Content Within rAAVs Impacts Performance in a Cell Assay-Dependent Manner," *Molecular Therapy—Methods & Clinical Development* 30 (2023): 288–302, <https://doi.org/10.1016/j.omtm.2023.07.007>.
23. E. M. Shitik, I. K. Shalik, and D. V. Yudkin, "AAV- Based Vector Improvements Unrelated to Capsid Protein Modification," *Frontiers in Medicine* 10 (2023): 1106085, <https://doi.org/10.3389/fmed.2023.1106085>.
24. B. Furuta-Hanawa, T. Yamaguchi, and E. Uchida, "Two-Dimensional Droplet Digital PCR as a Tool for Titration and Integrity Evaluation of Recombinant Adeno-Associated Viral Vectors," *Human Gene Therapy Methods* 30, no. 4 (2019): 127–136, <https://doi.org/10.1089/hgtb.2019.031>.
25. L. Tereshko, X. Zhao, J. Gagnon, et al., "A Novel Method for Quantitation of AAV Genome Integrity Using Duplex Digital PCR," *PLoS ONE* 18, no. 12 (2023): 0293277, <https://doi.org/10.1371/journal.pone.0293277>.
26. Y. Chen, S. Hu, W. Lee, et al., "A Comprehensive Study of the Effects by Sequence Truncation Within Inverted Terminal Repeats (ITRs) on the Productivity, Genome Packaging, and Potency of AAV Vectors," *Microorganisms* 12, no. 2 (2024): 310, <https://doi.org/10.3390/microorganisms12020310>.
27. H. Liu, Y. Zhang, M. Yip, et al., "Producing High-Quantity and High-Quality Recombinant Adeno-Associated Virus by Low-Cis Triple Transfection," *Molecular Therapy—Methods & Clinical Development* 32, no. 2 (2024): 101230, <https://doi.org/10.1016/j.omtm.2024.101230>.
28. D. A. Dalwadi, A. Calabria, A. Tiyaonchai, et al., "AAV Integration in Human Hepatocytes," *Molecular Therapy* 29, no. 10 (2021): 2898–2909, <https://doi.org/10.1016/j.ymthe.2021.08.031>.
29. O. Kondratov, D. Marsic, S. M. Crosson, et al., "Direct Head-to-Head Evaluation of Recombinant Adeno-Associated Viral Vectors Manufac-

tured in Human Versus Insect Cells,” *Molecular Therapy* 25 (2017): 2661–2675, <https://doi.org/10.1016/j.ymthe.2017.08.003>.

30. M. Janc, K. Zevnik, A. Dolinar, et al., “In-Depth Comparison of Adeno-Associated Virus Containing Fractions After CsCl Ultracentrifugation Gradient Separation,” *Viruses*. 16, no. 8 (2024): 1235, <https://doi.org/10.3390/v16081235>.

Supporting Information

Additional supporting information can be found online in the Supporting Information section.